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TP53 Signature Score Predicts Prognosis and Immune Response in Triple-negative Breast Cancer

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Abstract. *Background/Aim: Triple-negative breast cancer (TNBC) is considered a heterogeneous disease and achieving a pathological complete response (pCR) to neoadjuvant chemotherapy (NAC) is considered a surrogate biomarker of a favorable prognosis. Previously, the TP53 signature (TP53sig)-score, the expression profile of 33 genes, has been reported to predict the prognosis of all types of early-stage breast cancer. Herein, we analyzed whether the TP53sig-score can be used to subclassify a TNBC cohort and investigated the molecular biological characteristics of the higher TP53sig-score. Patients and Methods: Publicly available data from TCGA (RNA-sequence) and METABRIC (microarray) and expression data from real clinical specimens (NanoString Technologies) were used to explore the prognosis and molecular features of TNBC. Results: The high TP53sig-score group in the present study and the cohort in METABRIC tended to have a worse prognosis than the low TP53sig-score group (p=0.583 and 0.196, respectively). In both the pCR and non-pCR groups, the high TP53sig-score patients tended to have a poor prognosis (p=0.0739). Moreover, when the NAC response and TP53sig-score were combined, the five-year breast cancer-free rate among the four groups differed significantly (p=0.043). In addition, high TP53sig-score was related to gene ontology terms, such as "cell differentiation" and "innate immune response". Notably, this group had the potential to respond favorably to immunotherapy according to the tumor immune dysfunction and exclusion model. Conclusion: The combination of the response to NAC and the TP53sig-score in TNBC was able to predict an unfavorable prognosis. Furthermore, patients with a high TP53sig-score showed a favorable response to immunotherapy.*

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Key Words: Triple-negative breast cancer, gene signature, tumor immunity.

Triple-negative breast cancer (TNBC), characterized by estrogen receptor (ER)-negativity, progesterone receptor (PgR)-negativity, and human epidermal growth factor receptor-related 2 (HER2)-negativity, is known to have the worst prognosis among the breast cancer subtypes (1, 2). A pathological complete response (pCR) to neoadjuvant chemotherapy (NAC) using cytotoxic drugs, such as anthracyclines and taxane, is considered a surrogate biomarker of a favorable prognosis, and additional treatment after both NAC and surgery reportedly improves the prognosis of non-pCR patients (3, 4). Recently, pembrolizumab showed an improvement in the pCR rate for high-risk early TNBC in combination with chemotherapy (5). However, recurrences have been reported in patients who have achieved pCR, indicating the need for more accurate predictive and prognostic biomarkers (6, 7).

Originally, the *TP53* signature (*TP53*sig) was developed on the basis of various genes expressed in *TP53* structural mutant-type (MT) and wild-type (WT) breast cancers (8). The *TP53*sig is a continuous, non-parametric value that can be used as a risk score. Previously, we reported that the overall prognosis of all subtypes of breast cancer could be predicted by setting a risk score cut-off for all subtypes and classifying each under the *TP53*sig MT or WT types. When the*TP53*sig of all the subtypes was classified using the cut-off value used in previous reports, most cases of TNBC were of the *TP53*sig MT type, although *TP53*sig MT type did not fully match *TP53* somatic mutations (9). Furthermore, the molecular biology of the *TP53*sig was characterized by increased genome instability and promotion of cell cycle progression (9). Although each cancer subtype has already been examined in previous studies, TNBC has tumor

heterogeneity, and its response to NAC and prognosis vary widely, as described above. Accordingly, TNBC was classified by the *TP53*sig, then predictive value of the *TP53*sig-score was assessed, and the NAC response and prognosis were analyzed according to the hypothesis that the *TP53*sig can be subclassified in detail even in a TNBC cohort. The present study also examined the molecular biological characteristics of patients with TNBC with a high *TP53*sig-score to consider new strategies of treatment.

Patients and Methods

Patient data from public databases. The cancer genome atlas program (TCGA) dataset was downloaded *via* cBioportal and UCSC Xena (10-12). The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset was obtained from cBioportal.

Our own data. The medical records of 71 patients with TNBC who received NAC between January 2006 and December 2014 at Tokyo Metropolitan Cancer and Infectious Diseases Center at Komagome Hospital in Japan were retrospectively reviewed. We defined TNBC as ER <1%, PgR <1%, and HER2-negative status. HER-2 negative means a score of 0 or 1 by immunohistochemical analysis or the absence of HER2-amplification by fluorescence in situ hybridization with an immunohistochemistry score of 2. Samples from 60 patients were analyzed for gene expression and clinicopathological data. pCR is defined as no invasive residual tumor in breast and lymph nodes after NAC.

The patient data were collected in accordance with the ethical guidelines for medical and health research involving human subjects. Our protocol was approved by the institutional ethical committee (November 10th, 2018/No. 2182) and conducted according to the principles of the Declaration of Helsinki. All the patients provided informed consent in the form of opt-out.

RNA extraction. Glass slide specimens with 10-μm thick sections of formalin-fixed paraffin-embedded (FFPE) tissue block that were collected by core-needle biopsy or vacuum-assisted biopsy at the diagnosis were prepared. Total RNA was extracted from the samples using RNeasy mini kit (Qiagen, Hilden, Germany).

Gene expression analysis via nCounter. RNA quality was monitored using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Except samples of low yields of RNA extraction, we analyzed 60 FFPE specimens using digital quantification *via* the nCounter (NanoStrings Technologies, Seattle, WA , USA). A set of sixty genes including five internal control genes was used as the *TP53* signature gene set for nCounter. Following the manufacturer's instructions, we measured the expression values of the 33 genes using nCounter with 200 ng of total RNA extracted from the FFPE samples.

*TP53*sig-*score*. The *TP53*sig-score was calculated using microarray expression data or nCounter according to the following formula. *TP53*sig-score=(sum of the count of 24 genes up-regulated in tumors with a *TP53* mutation)/(sum of the count of nine genes down-regulated in tumors with a *TP53* mutation).

Statistical analysis using clinical information. The association between various clinicopathological parameters and the *TP53*sig-score as determined using nCounter or microarray expression data was evaluated using the chi-square test or one-way analysis of variance test. The *t*-test was used to analyze the expression data and the status of the *TP53*sig-score. The relapse-free survival (RFS) and breast cancer-free survival (BCFS) rates were calculated using the Kaplan–Meier method and evaluated using the log-rank test. RFS was defined as the period from the date of operation to the date of recurrence, and BCFS was defined as the period from the date of operation to the date of invasive breast cancer recurrence. For all statistical analyses, $p<0.05$ was considered to indicate statistical significance. All statical analyses were conducted using R ver3.32 (The R Foundation for Statistical Computing, Vienna, Austria) and EZR ver1.54 (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (13).

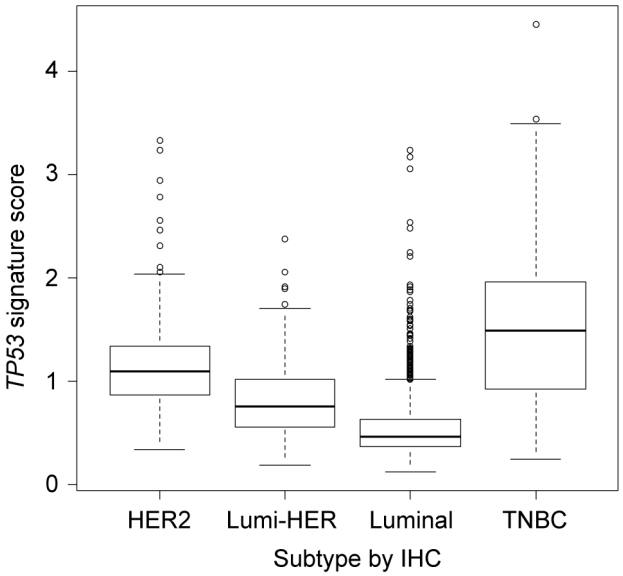

Figure 1. *The TP53 signature (TP53sig) score per breast cancer subtype from METABRIC. The triple negative breast cancer (TNBC) cohort had a higher TP53sig score than the other subtypes (p<0.001).*

Gene ontology enrichment analysis. Gene Ontology (GO) enrichment analysis was performed using David ver6.8 (14, 15). In the function annotation chart obtained from DAVID, GO terms with $p<0.1$ were considered to indicate statistical significance.

TIDE evaluation. Tumor Immune Dysfunction and Exclusion (TIDE) evaluation was performed *via* the website platform (16, 17). The TIDE score computed for each tumor sample can serve as a surrogate biomarker to predict the response to immune checkpoint blockade, including anti-PD1 and anti-CTLA4, for melanoma and NSCLC (16, 17).

Data availability. The data that support the findings of the present study are openly available in Gene Expression Omnibus at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE190275, reference number GSE190275.

Table I. *Clinicopathological patient characteristics per group*.

Samples	Our own data (n=60)						TCGA (n=50)						METABRIC (n=279)					
	Total	%	High *TP53*sig	%	Low *TP53*sig	%	Total	%	High *TP53*sig	%	Low *TP53*sig	%	Total	%	High *TP53*sig	%	Low *TP53*sig	%
	60	100%	30	50%	30	50%	50	100%	25	50%	25	50%	279	50%	139	50%	140	50%
Age at diagnosis	51.5 y.o. (28-83)		50 y.o. (28-63)		53 y.o. (34-83)		52 y.o. (26-82)		51 y.o. (37-82)		52 y.o. (26-82)		54 y.o. (26-96)		53 y.o. (26-84)		55 y.o. (28-96)	
Sex																		
Female	60	100%	30	100%	30	100%	50	100%	25	100%	25	100%	279	100%	139	100%	140	100%
*TP53*sig score	2,163 (0.521-4.302)		3,014 (2.205-4.302)		1,321 (0.521-2.121)		0,788 (0.380-0.792)		0,96 (0.792-1.22)		0,687 (0.380-0.784)		1,221 (0.346-1.961)		1,346 (1.221-1.961)		1,037 (0.346-1.221)	
Tumor size (T)																		
1	7	11%	2	7%	5	17%	13	26%	7	28%	6	24%	77	27%	32	23%	45	32%
2	36	60%	17	57%	19	63%	28	56%	17	68%	11	44%	172	62%	89	64%	83	59%
3	13	22%	10	33%	3	10%	6	12%	1	4%	5	20%	28	10%	17	12%	11	8%
4	4	7%	1	3%	3	10%	2	4%	0	0%	2	8%	0	0%	0	0%	0	0%
X	0	0%	0	0%	0	0%	1	2%	0	0%	1	4%	2	1%	1	1%	1	1%
Node (N)																		
Negative	20	33%	6	20%	14	47%	31	62%	17	68%	14	56%	133	48%	63	45%	70	50%
Positive	40	67%	24	80%	16	53%	19	38%	8	32%	11	44%	146	52%	76	55%	70	50%
NAC response																		
pCR	21	35%	13	44%	8	26%	NA		NA		NA		NA		NA		NA	
Non-pCR	39	65%	17	56%	22	74%	NA		NA		NA		NA		NA		NA	
NAC regimen																		
AC-T*	53	89%	28	93%	NA		NA		NA		NA		NA		NA		NA	
Other	7	11%	2	7%	NA		NA		NA		NA		NA		NA		NA	
Event																		
Death	10	16%	7	24%	3	11%	8	16%	3	12%	5	20%	150	54%	73	53%	77	55%
Recurrence	13	21%	9	30%	4	13%	NA		NA		NA		117	42%	57	41%	60	43%

There was no significant difference between high *TP53*sig and low *TP53*sig in each cohort. *AC→T: Anthracycline followed by taxane (Docetaxel or Paclitaxel). NAC: Neoadjuvant chemotherapy; TCGA: The Cancer Genome Atlas; METABRIC: Molecular Taxonomy of Breast Cancer International Consortium.

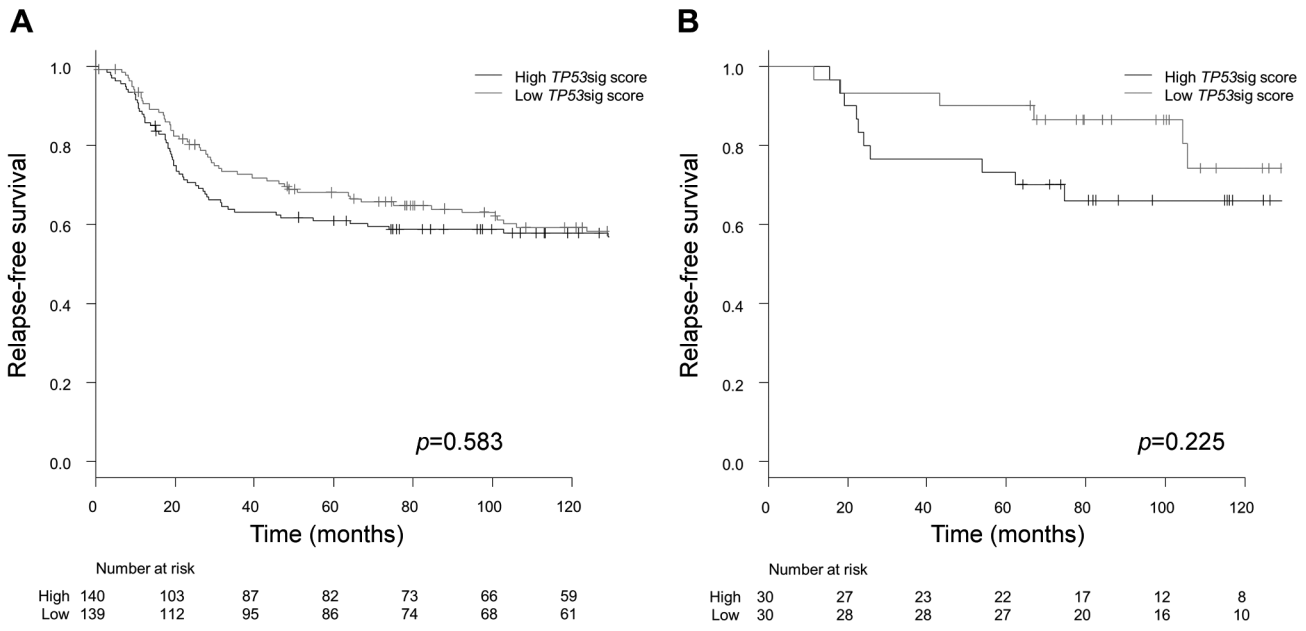

Figure 2. *Relapse-free survival analysis of the high and low TP53 signature (TP53sig) score groups in METABRIC (A) and our own data (B). Both cohorts tended to have a worse prognosis in the high TP53sig score population than in a low TP53sig score population, but the difference was not significant [p=0.583 (A) and p=0.196 (B)].*

Results

TP53sig-score for all breast cancer subtypes. We extracted 1,904 stage I-III breast cancers from METABRIC and classified ER+ or PgR+ and HER2- as the luminal-type, ER+ or PgR+ and HER2+ as the luminal-HER2 type, ER–, PgR–, and HER2+ as the HER2-enriched type, and ER–, PgR–, and HER2– as the TNBC type according to the results of ER/PgR/HER2 based on immunohistochemical (IHC) analysis of the four groups. When the *TP53*sig was compared in the last group, TNBC was found to have the highest score (Figure 1; $p<0.001$).

Patient characteristics and survival analysis in TNBC (METABRIC and our data). In the present study, we analyzed our own data that is the TNBC cohort previously treated with chemotherapy and obtained TNBC cohorts from TCGA and METABRIC. we analyzed our own data, including the TNBC-with-NAC and TNBC cohorts obtained from TCGA and METABRIC. As Table I shows, the *TP53*sig was calculated on the basis of expression data or microarray data, and the median of the *TP53*sig-score in each cohort was used to divide into the high and low *TP53*sig groups. Clinicopathological patient characteristics of these two groups did not differ significantly in either dataset. Both our own cohort and the METABRIC cohort tended to have a worse prognosis in the low *TP53*sig-score than in the high *TP53*sig-score population, although the difference was not significant (Figure 2; $p=0.583$ and $p=0.196$, respectively).

Survival analysis when combining the TP53sig-score and NAC response. We analyzed the relationship between the *TP53*sig-score and NAC response in patients with TNBC. In terms of predicting the NAC response, *MKI-67* levels were higher in pCR patients (Figure 3A; $p=0.0453$) whereas the *TP53*sig-score tended to be only slightly higher in the pCR patients although the difference was not statistically significant (Figure 3B; $p=0.153$). In both the pCR and non-pCR patients, patients with a high *TP53*sig-score group tended to have a poor prognosis (Figure 4A; $p=0.0739$). In contrast, *MKI-67* scores showed no such tendency (Figure 4B; $p=0.734$). In addition, when the *TP53*sig-score was combined with the NAC response, the five-year breast cancer-free rate was found to be significantly different among the four groups (Table II and Table III; $p=0.043$).

Differentially expressed genes (DEGs) in the low and high TP53sig-score groups. DEGs in the high and low *TP53*sig-score groups in the TCGA were identified, and 498 DEGs with $p<0.1$ and less than two-fold change were detected. Many of these genes were related to GO terms, such as "cell differentiation" (*e.g.*, *DAPL1, USH1C, TEX15, DLK1, EDAR, SLC22A16, ZIC2, NHLH1, ELF5, SFRP5, SMOC1, PRAME, NKX2-5, KRT6A*) and "innate immune response"

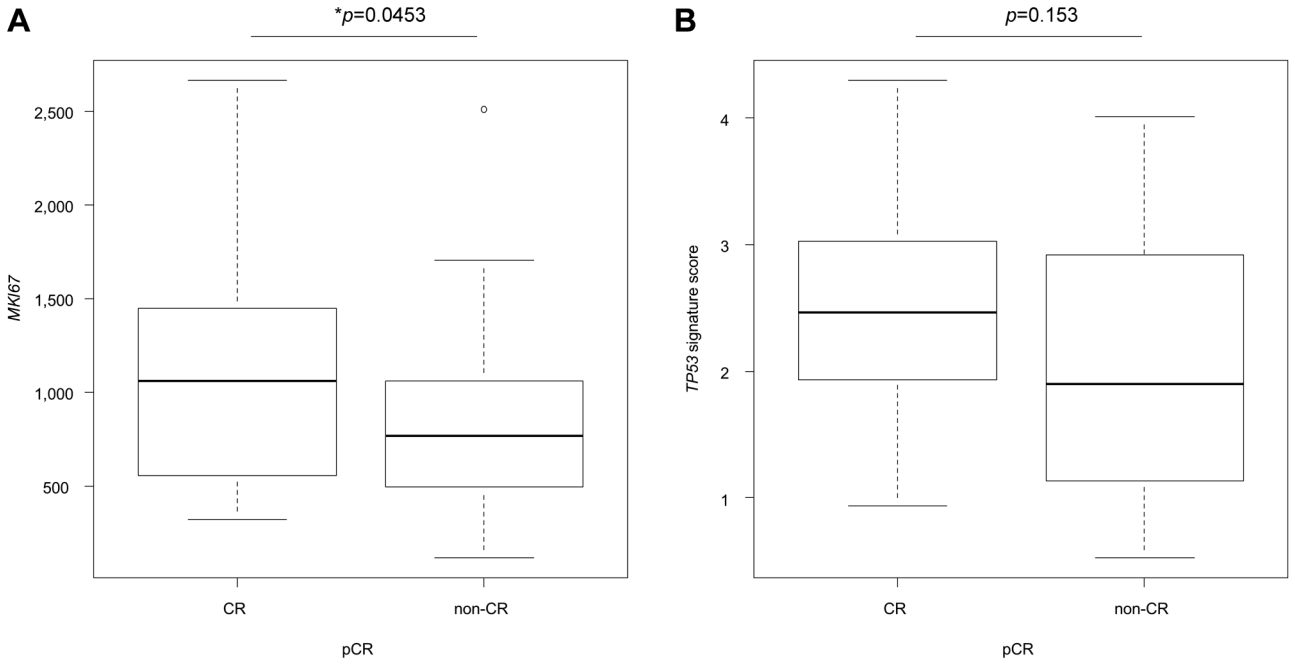

Figure 3. *Association of neoadjuvant chemotherapy effect with MKI-67 status (A) and TP53 signature (TP53sig) score (B). MKI-67 level was higher in pathological complete response [pCR patients (p=0.0453)], whereas the TP53sig score tended to be slightly higher in pCR patients (p=0.153).*

Table II. *Number of 5-year breast cancer events among each group with MKI-67.*

	pCR/High *MKI-67*	pCR/Low *MKI-67*	Non-pCR/High *MKI-67*	Non-pCR/Low *MKI-67*
Recurrence	0	1	6	4
No recurrence	12	8	12	17

Table III. *Number of 5-year breast cancer events among each group with TP53sig. The five-year breast cancer-free rates in the four groups were found to be significantly different (p=0.043).*

	pCR/High *TP53*sig	pCR/Low *TP53*sig	Non-pCR/High *TP53*sig	Non-pCR/Low *TP53*sig
Recurrence	1	0	7	3
No recurrence	12	8	10	19

(*e.g.*, *DEFB1, IL27, MARCO, CLEC4C, SLPI, CLEC6A, NLRP2, LCN2, CD300E, IL36RN, S100A9, S100A8, S100A7, IGHV1OR21-1*) in the high *TP53*sig-score group.

Evaluating tumor immunity environment using TIDE. Tumor immunity was assessed using TIDE, a tool for predicting the effects of an immune checkpoint inhibitor (ICI) and was compared between the high and low *TP53*sig-score groups. In the METABRIC cohort (Figure 5A-F), high *TP53*sig-score group was associated with a higher proportion of responders (p=0.00253), high CD 274 related to PD-L1 expression (p<0.0454), low T cell exhaustion (p<0.001), low expression profiles of cancer-associated fibrosis (CAF), and tumor-associated macrophages (TAMs) (p<0.001 and

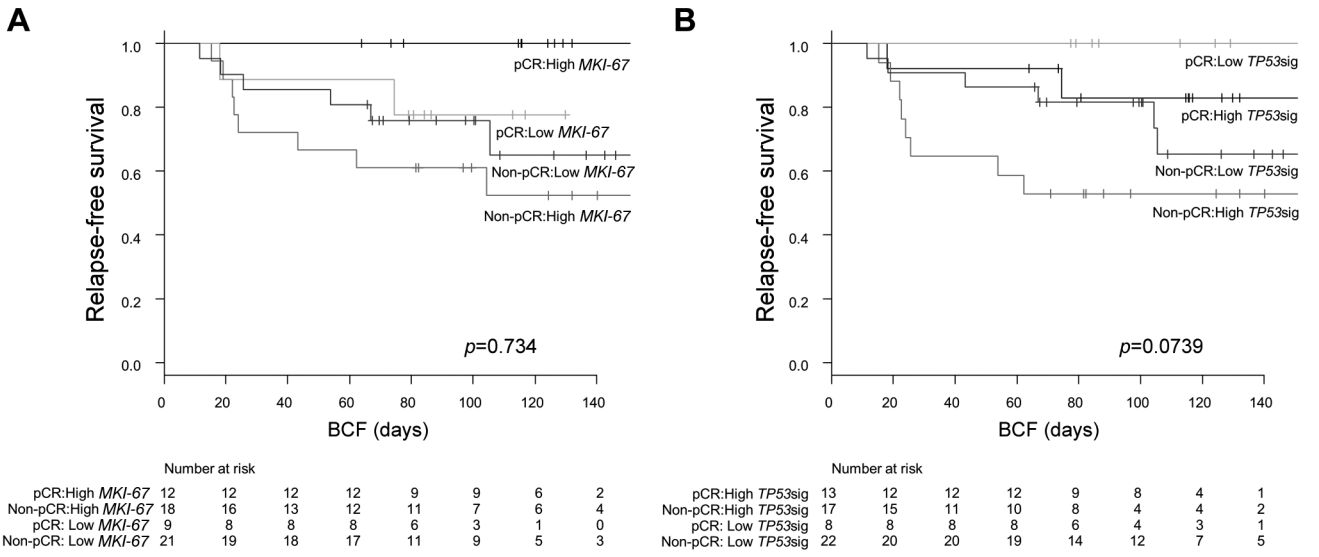

Figure 4. *Relapse-free survival (RFS) analysis for each group according to neoadjuvant chemotherapy (NAC) response and MKI-67 status (A). RFS analysis according to NAC response and TP53 signature (TP53sig) score (B). In both pathological complete response (pCR) and non-pCR patients, patients in the high TP53sig score group tended to have a poor prognosis (Figure 4A; p=0.0739).*

p=0.0124, respectively). On the other hand, in high *TP53*sig-score group, an expression profile of myeloid-derived suppressor cells (MDSCs) was significantly high ($p<0.001$). The TCGA cohort (Figure 5G-L) showed a similar trend to that of the METABRIC cohort, but except for CAF, the difference was not statistically significant.

Discussion

In the present study, TNBC was associated with a high *TP53*sig-score and the high *TP53*sig-score group was in turn associated with a poorer prognosis compared to the low *TP53*sig-score groups. Regardless of the NAC response, the high*TP53*sig-score group had a poor prognosis. The *TP53*sig was developed as a gene signature to predict structural mutations in *TP53*, but it was also found to reflect aspects of the tumor immune environment, such as TIDE, PD-L1, CAF, TAMs, and MDSCs.

Previous studies reported that *TP53*sig-MT was associated with the promotion of cell cycle progression, and that the expression signature, rather than the structural mutation of *TP53,* was useful in predicting the effect of fluorouracil, epirubicin and cyclophosphamide (FEC) followed by paclitaxel in all breast cancer subtypes (9, 18). In addition, the *TP53* MT type population reportedly has a high degree of nuclear atypia and *MKI-67*. In the present study, most of the TNBC *TP53* structural mutations and studies of the usual *TP53* MT type population showed that the *TP53*sig-score was inferior to *MKI-67*, which better reflects the cell cycle, but that a combination of the NAC response and *TP53*sig was better able to predict the prognosis.

The present study found that there was a high proportion of MDSCs in the high *TP53*sig-score group. MDSCs increase in cancer, inflammation, and infections. Furthermore, they inhibit the T-cell response and has been identified as a predictor of resistance to anticytotoxic T-lymphocyte-associated protein (CTLA4) antibodies in melanoma (19, 20). MDSCs are also reportedly a poor prognostic marker of breast cancer, suggesting that it may be one of the causes of the poor prognosis of the high *TP53*sig-score population (21). *In vivo* studies of breast cancer have reported that doxorubicin induced a reduction in MDSCs and that in other cancer species, 5-fluorouracil (5-FU) administration induced a decrease in MDSCs (22, 23). Based on these results, the 5-FU and doxorubicin regimens may be a practical therapeutic option for the high-MDSC population, and of the 5-FU regimens, capecitabine may have the potential to be an effective treatment for high-risk TNBC, including cases with non-pCR (3).

The MT-type *TP53* signature is reportedly associated with a high PD-L1 level (9). In addition, the structural mutation of *TP53* itself is also associated with PD-L1 expression, and genomic instability is a predictor of ICI efficacy (24-27). ICIs are also thought to be effective against TNBC, especially in cases with a high *TP53*sig-score.

The anti-PD-L1 and anti-PD-1 antibodies are reportedly useful not only against TNBC metastases but also in the perioperative period (5, 28-30). PD-L1 expression in IHC assays and microsatellite instability is the only predictive

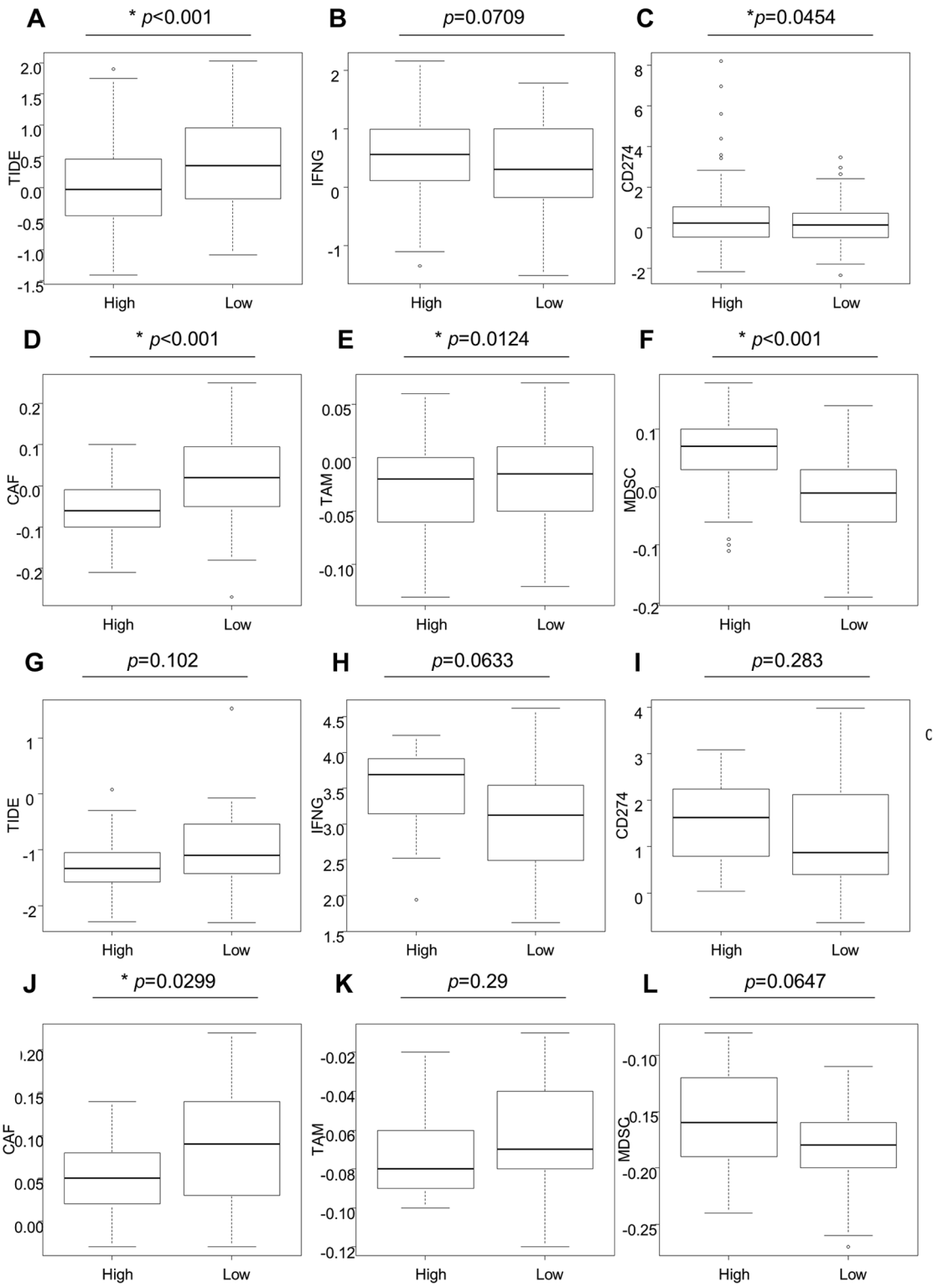

Figure 5. *Tumor immunity evaluation in high and low TP53 signature (TP53sig) groups based on data from METABRIC (A-F) and TCGA (G-L) compared with tumor immune dysfunction and exclusion (TIDE) (A, G), interferon-gamma (B, H), CD274 (C, I), CAF (D, J), TAM (E, H), and MDSC (F, L). In the METABRIC cohort, in the high TP53sig group, the TIDE score predictive of T cell exhaustion was significantly low (p<0.001), the CD274 scores related to PD-L1 expression were high (p<0.0454), and cancer-associated fibrosis (CAF) and tumor-associated macrophages (TAMs) were also significantly low (p<0.001 and p=0.0124). On the other hand, myeloid-derived suppressor cells (MDSCs) were significantly high (p<0.001). The TCGA cohort (G-L) showed a similar trend to that of the METABRIC cohort, but except for CAF, the difference was not statistically significant.*

biomarker of efficacy, and thus more detailed biomarkers are needed (29). TIDE was developed as a tool to calculate T cell exhaustion as well as PD-L1 expression and predict the effect of ICIs and expression profiles of CAFs, TAMs, and MDSCs. CAFs and TAMs have immunosuppressive roles as they reduce T cell responses and regulate the killing effect of T cells and natural killer cells (31-33). The present study suggested that the high *TP53*sig-score and usual non-pCR population in TNBC had a poor prognosis whereas the high *TP53*sig-score population had a low TIDE score and T-cell function. High PD-L1 expression also predicted a favorable response to ICIs, and patients with this feature are considered good candidates for ICI therapy.

In the present study, ICI efficacy was not directly investigated, nor was the tumor immune environment evaluated owing to the limited data available from nCounter for patients receiving NAC. However, two, large, public databases, TCGA and METABRIC, found comparable results for the tumor immune environment, suggesting that the high *TP53*sig-score group among patients with TNBC may be good candidates for ICI therapy.

In conclusion, combining the NAC response with the *TP53*sig-score was able to predict a poor prognosis in patients with TNBC, and the high *TP53*sig-score population was found to have a favorable immunotherapy response.

Conflicts of Interest

The Authors have no conflicts of interest directly relevant to the content of this article.

Authors' Contributions

M.O, S.Y. designed the study. M.O., T.A. and SI.H. reviewed clinical and pathological information. M.H. and S.Y. advised on the RNA extraction protocol and how to use the nCounter. M.O. and S.Y. performed the statistical analyses. M.O. wrote the manuscript. S.Y. and S.K. critically revised the manuscript. All Authors read and approved the manuscript.

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